Technical Overview: Multiplexed Amplicon Library Preparation Using SMRTbell Express Template Prep Kit 2.0

Sequel System ICS v8.0 / Sequel Chemistry 3.0 / SMRT Link v9.0
Sequel II System ICS v9.0 / Sequel II Chemistry 2.0 / SMRT Link v9.0
Sequel Ile System ICS v10.0 / Sequel II Chemistry 2.0 / SMRT Link v10.0
Multiplexed Amplicon Library Preparation Using SMRTbell Express Template Prep Kit 2.0

1. Multiplexed Amplicon Library Sample Preparation Workflow Overview
2. Amplicon Sample QC Requirements and SMRTbell Express TPK 2.0 Handling Recommendations
3. Multiplexed Amplicon Library Preparation Using PacBio Barcoded Overhang Adapters (BOA)
5. Multiplexed Amplicon Library Sequencing Workflow Recommendations
6. Multiplexed Amplicon Data Analysis Recommendations
7. Multiplexed Amplicon Library Example Sequencing Performance Data
8. Technical Documentation & Applications Support Resources

Appendix 1: Multiplexed Amplicon Library Preparation Using PacBio Barcoded M13 Primers
Appendix 2: Sample Preparation Recommendations for HLA Amplicon SMRT Sequencing
MULTIPLEXED AMPLICON SEQUENCING: HOW TO GET STARTED

**Application-Specific Best Practices Guide**

**Application-Specific Procedure & Checklist**

**Application Consumable Bundle Purchasing Guide**

**Library Construction, Sequencing & Analysis**

- **PCR Amplicon Generation**
  - Barcoding Options: Barcoded Target-Specific Primers or Barcoded Universal Primers (BUP)

- **Library Construction** (SMRTbell Express TPK 2.0)
  - Barcoding Options: Barcoded Overhang Adapters (BOA)

- **HiFi Sequencing**
  - Generate Up To 3 Million HiFi Reads per SMRT Cell 8M

- **Data Analysis** (SMRT Link)
  - CCS Analysis
  - Demultiplexing Analysis

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**Application Brief: Targeted sequencing for amplicons – Best Practices** (BP105-071919)

Summary overview of application-specific sample preparation and data analysis workflow recommendations

**Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplexing Amplicons** (101-791-700)

**Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplex SMRT Sequencing** (101-791-800)

**Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing** (101-921-300)

**PacBio Application Consumable Bundle Purchasing Guide** (PG100-082620)

Purchasing Guide enables users to easily order required consumables needed to prepare a SMRTbell library to run a specific type of application on the Sequel II and IIe Systems*

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* Application Consumable Bundles include reagents for library construction, primer annealing and polymerase binding. Core PacBio-branded SMRT Sequencing consumables (SMRT Cells, Sequencing Kits & SMRT Oil), plastics and other 3rd-party reagents are not included in the application bundles.
Multiplexed Amplicon Library Sample Preparation Workflow Overview
BARCODING OPTIONS FOR MULTIPLEXED AMPLICON SEQUENCING

### Barcoded Target-Specific Primers

- **Customer-Supplied Primers**
- **PacBio-Supplied BOAs**
- **Customer-Supplied BOAs**

- **Target-specific primers** are tailed with PacBio 16-bp barcodes to produce **symmetrically** or **asymmetrically** barcoded samples using a 1-step PCR method.
- Can be used for small or large projects when validation of barcoded target-specific primers will be performed at the start of a project.

### Barcoded Overhang Adapters (BOA)

- **PacBio-Supplied BOAs**
- **Customer-Supplied BOAs**

- PacBio 16-bp barcodes are added to amplicons through ligation of barcoded overhang SMRTbell adapters to produce **symmetrically** barcoded samples. (Procedure: PN 101-791-700)
- Two sets of 8 barcoded overhang adapters are commercially available from PacBio (PN 101-628-400/500)
- Recommended for smaller projects (≤96 samples) using validated PCR systems and off-the-shelf assays.

### Barcoded Universal Primers (BUP)

- **PacBio-Supplied Barcoded Universal Primers**
- **Customer-Supplied Barcoded Universal Primers (e.g., Barcoded M13 Primers)**

- PacBio 16-bp barcodes are added to amplicons through a 2-step PCR method using Universal Sequence-tagged target-specific primers and Universal Sequence-tagged 16-bp barcoded primers. (Procedure: PN 101-791-800 / 101-921-300)
- For **symmetric** barcoding of ≤96 amplicon samples, we recommend using our 96 Barcoded Universal Primers kit (PN 101-629-100)
- For **asymmetric** barcoding of >96 amplicon samples, we recommend using the Barcoded M13 Primer approach with custom-ordered barcoded M13 primers (see Appendix 1).

Learn more about PacBio's barcoding solutions: [Visit our products and services](https://www.pacb.com/products-and-services/consumables/multiplexing-kits/)

For more information, visit [PacBio's Smrt Science Smrt Sequencing Multiplexing](https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/)
WORKFLOW SUMMARY OVERVIEW: MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO-BARCODED TARGET-SPECIFIC PRIMERS

Barcoded amplicons are generated via a 1-step PCR. Once the PCR products are barcoded, they can be pooled into a single tube for SMRTbell library construction.

1-Step PCR Amplicon Generation with Customer-Supplied Target-Specific Primers
- Perform PCR using Target-Specific Primers tailed with PacBio 16-bp barcode sequences
- Minimum required input DNA mass per pooled sample for library construction is ≥250 ng (dependent on insert size)

SMRTbell Library Construction (4 hrs)
- Pool barcoded amplicon samples as described in the ‘Sample Pooling’ step on page 7 of the Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplexing Amplicons (PN 101-791-800) and proceed with library construction

HiFi Sequencing
- Follow Quick Reference Cards for primer annealing, polymerase binding, complex cleanup and sample loading
- Generate up to 3 million HiFi reads per SMRT Cell 8M* (dependent on insert size)

Data Analysis
- Demultiplex barcodes using SMRT Link
- Analyze HiFi data using SMRT Link or other third-party tools

* Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.
WORKFLOW SUMMARY OVERVIEW: MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO-BARCODED OVERHANG ADAPTERS (BOA)

A barcode is introduced to each amplicon via ligation with a hairpin adapter containing a 16-bp PacBio barcode sequence. Once barcoded, amplicons can be pooled as a single SMRTbell library sample for sequencing.

**Input PCR Amplicon QC**
- Minimum required input DNA mass per amplicon for library construction is 10 ng

**SMRTbell Library Construction (<5 hrs)**
- Follow Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplexing Amplicons (PN 101-791-700) and pool samples
- Use PacBio Barcoded Overhang Adapter Kit 8A//8B (PN 101-628-400/500) to multiplex up to 16 samples
- If multiplexing >16 samples, order barcoded overhang adapters directly from a third-party oligo synthesis provider

**HiFi Sequencing**
- Follow Quick Reference Cards for primer annealing, polymerase binding, complex cleanup and sample loading
- Generate up to 3 million HiFi reads per SMRT Cell 8M* (dependent on insert size)

**Data Analysis**
- Demultiplex barcodes using SMRT Link
- Analyze HiFi data using SMRT Link or other third-party tools

* Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.
Barcoded amplicons are generated via a 2-step PCR. Once the PCR products are barcoded, they can be pooled into a single tube for SMRTbell library construction.

**2-Step PCR Amplicon Generation with Barcoded Universal Primers or Barcoded M13 Primers**

**SMRTbell Library Construction (4 hrs)**
- Pool barcoded amplicon samples and proceed with library construction
- Minimum required input DNA mass per pooled sample for library construction is ≥250 ng

**HiFi Sequencing**
- Follow *Quick Reference Cards* for primer annealing, polymerase binding, complex cleanup and sample loading
- Generate up to 3 million HiFi reads per SMRT Cell 8M* (dependent on insert size)

**Data Analysis**
- Demultiplex barcodes using SMRT Link
- Analyze HiFi data using SMRT Link or other third-party tools

* Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.

PacBio HiFi reads achieve >99.9% accuracy
Amplicon Sample QC Requirements and SMRTbell Express TPK 2.0 Handling Recommendations
BEST PRACTICES FOR GENERATING HIGH-QUALITY PCR AMPLICONS

- **Clean, target-specific PCR products** are extremely important for obtaining high-quality sequence data.

- Non-specific products can represent a substantial percentage of the sequencing reads if they are not removed.

- To minimize their presence, consider the recommendations described in the following sections for generating **high-quality amplicons** suitable for SMRTbell library preparation and sequencing.
BEST PRACTICES FOR GENERATING HIGH QUALITY PCR AMPLICONS (CONT.)

1. Begin with high-quality nucleic acids and work in a clean environment.
   a. For targeted sequencing of genomic DNA (gDNA) samples, starting with high-quality DNA will result in better performance during sequencing.
      ▪ Technical Note: DNA Prep (PN TN101-061920) provides recommendations, tips and tricks for genomic DNA extraction as well as assessing and preserving the quality and size of your DNA sample to be used for PacBio HiFi sequencing
   b. If extracted nucleic acids must be stored, freeze at high concentrations in appropriately-buffered solutions.
   c. To minimize possible contamination and degradation caused by multiple freeze/thaw cycles, sub- aliquot DNA into smaller volumes for storage.
   d. Set up PCR reactions in an environment free from sources of non-specific primer and template contaminants, ideally a laminar flow hood, using dedicated pre-PCR pipettor, tips and reagents.
2. Use PCR reagents and conditions for generating target-specific, full length amplicons.
   a. Use the highest-fidelity polymerase compatible with your PCR amplification system.
   b. Use desalted or HPLC-purified oligo primers; damaged bases at the ends of the amplicons cannot be repaired by DNA Damage Repair enzymes.
   c. Optimize PCR conditions to minimize total time spent at high (>65°C) temperatures, particularly during denaturation.
   d. PCR extension time should be long enough to ensure complete extension, taking into consideration the polymerase used and target amplicon size.
      a. For mixed samples with similar targets, it is important to complete extension at every step to avoid generating chimeric products in subsequent steps.
      b. As a general guideline, use extension times of one minute per 1000 base pairs (e.g., 3 minutes for a 3 kb product).

3. **Use the lowest number of cycles** required for obtaining adequate yields (ng) of PCR products to proceed with SMRTbell library construction. Avoid over-amplification.

4. If non-specific products are present, **optimize PCR conditions or perform AMPure PB bead-based size selection** to enrich for PCR amplicons with the desired target size.
EVALUATION OF PCR AMPLICON DNA CONCENTRATION

- For accurate quantification of PCR samples to be used in multiplexed amplicon library preparation workflows, PacBio recommends using the **Qubit fluorometer** and **Qubit dsDNA High Sensitivity (HS) assay reagents** (Thermo Fisher Scientific)
  - Qubit dsDNA HS assay quantitation range: 0.2 – 100 ng
  - **Note:** When measuring very low DNA concentrations of short-amplicon samples (<1 kb library insert size), it may be helpful to **increase the sample aliquot volume above 1 µL** (up to 20 µL) in order to ensure sufficient assay sensitivity.

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**Qubit™ dsDNA HS Assay Kit**

**Qubit 4 Fluorometer**

[https://www.thermofisher.com/order/catalog/product/Q33230#Q33230](https://www.thermofisher.com/order/catalog/product/Q33230#Q33230)
EVALUATION OF PCR AMPLICON DNA PURITY

- Amplicon DNA purity can be assessed through UV-spectrophotometry using a Nanodrop spectrophotometer
- For pure DNA, A260/280 ratio is typically ~1.8 or higher and A260/230 ratio is ≥2.0 or higher
- For samples with ratios that fall outside the expected optimal values, PacBio recommends performing an AMPure bead purification step followed by re-assessment of quantity and purity of the DNA sample
  - See Technical Note: DNA Prep (PN TN101-061920) for recommended DNA cleanup procedure

260/280 Ratio

- A low A260/A280 ratio may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. Sometimes it may be caused by a very low concentration of nucleic acid.
- High 260/280 ratios are not normally indicative of an issue.

260/230 Ratio

- A low A260/A230 ratio may be the result of:
  - Carbohydrate carryover (often a problem with plants).
  - Residual phenol from nucleic acid extraction.
  - Residual guanidine (often used in column-based kits).
  - Glycogen used for precipitation.

PacBio recommends only proceeding with DNA samples that have an absorbance A260/A280 ratio between 1.8 – 2.0 and a A260/A230 ratio between 2.0 – 2.5.
EVALUATION OF PCR AMPLICON DNA SIZE DISTRIBUTION

- It is important to accurately assess the sizes of the amplicons that are being multiplexed before preparing SMRTbell libraries for sequencing.

- For sizing QC of amplicons, visualize an aliquot of each PCR reaction using an Agilent Bioanalyzer System, Agilent Fragment Analyzer System, Agilent Femto Pulse System or manual agarose gel electrophoresis with appropriate markers or ladders.

- If off-target/non-specific products are present, optimize PCR conditions or perform one or more rounds of AMPure PB bead-based purification to enrich for PCR amplicons with the desired target size.
  - If the contaminating bands are quite close in size or larger than the desired amplicon, or for any contaminants >1.5 kb, a gel-based size selection method is recommended.

PacBio recommends pooling amplicons of similar size (within approx. +/- 15% of the mean size) for sequencing on a single SMRT Cell.
RECOMMENDATIONS FOR AMPURE PB BEAD PURIFICATION OF AMPLICON SAMPLES

- It is highly recommended to purify amplicons before SMRTbell library construction to remove PCR reagents, buffers, primer dimers or short non-specific PCR products.

- Depending on the amplicon size, the required concentration of AMPure PB beads for purification varies.

- Use the table below to determine the appropriate concentration of AMPure PB beads to use for your specific sample.

### AMPURE PB BEAD RECOMMENDATIONS BASED ON AMPLICON SIZE

<table>
<thead>
<tr>
<th>AMPLICON SIZE</th>
<th>AMPURE PB BEAD RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 – 500 bp</td>
<td>1.8X</td>
</tr>
<tr>
<td>500 – 1000 bp</td>
<td>1.0X</td>
</tr>
<tr>
<td>1 – 3 kb</td>
<td>0.6X</td>
</tr>
<tr>
<td>3 – 10 kb</td>
<td>0.45X</td>
</tr>
<tr>
<td>15 kb</td>
<td>0.45X</td>
</tr>
</tbody>
</table>
SMRTBELL EXPRESS TEMPLATE PREP KIT 2.0 REAGENT HANDLING RECOMMENDATIONS

- Several reagents in the kit are sensitive to temperature and vortexing

- PacBio highly recommends:
  - Never leaving reagents at room temperature
  - Working on ice at all times when preparing master mixes
  - Finger tapping followed by a quick-spin prior to use

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>WHERE USED</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Damage Repair Mix v2</td>
<td>DNA Damage Repair</td>
</tr>
<tr>
<td>End Prep Mix</td>
<td>End-Repair/A-tailing</td>
</tr>
<tr>
<td>Overhang Adapter v3</td>
<td>Ligation</td>
</tr>
<tr>
<td>Ligation Mix</td>
<td>Ligation</td>
</tr>
<tr>
<td>Ligation Additive</td>
<td>Ligation</td>
</tr>
<tr>
<td>Ligation Enhancer</td>
<td>Ligation</td>
</tr>
</tbody>
</table>
Multiplexed Amplicon Library Preparation Using PacBio Barcoded Overhang Adapters (BOA)
PROCEDURE & CHECKLIST – PREPARING SMRTBELL LIBRARIES USING PACBIO BARCODED OVERHANG ADAPTERS FOR MULTIPLEXING AMPICONS

- This protocol (PN.101-791-700) describes a workflow for constructing symmetrically-barcoded SMRTbell libraries from up to 96 PCR amplicon samples using **PacBio Barcoded Overhang Adapters (BOA)** for multiplexed sequencing on the Sequel, Sequel II and Sequel IIe System (Sequel Systems).

- Protocol document contains:

  1. Best practices recommendations for generating high-quality PCR products for PacBio sequencing and input PCR amplicon QC
  2. General recommendations for planning multiplexing experiments using Barcoded Overhang Adapters
  3. Instructions for constructing **symmetrically barcoded SMRTbell libraries** with input PCR amplicons using PacBio’s SMRTbell Express Template Prep Kit 2.0 and PacBio Barcoded Overhang Adapter Kit 8A/8B (PN.101-628-400/500) or customer-supplied barcoded overhang adapters*
  4. Sample setup guidance for preparing multiplexed amplicon SMRTbell libraries for sequencing on the Sequel Systems

- For additional guidance on preparing HLA amplicon samples for multiplexed SMRT Sequencing, see **Reference Guide – Human Leukocyte Antigen (HLA) Sequencing** (PN.102-066-200) and Appendix 2 of this presentation

* Barcoded Overhang Adapter Kits 8A & 8B support multiplexing of up to 16 samples. If multiplexing projects require more than 16 samples (up to 96 samples), we offer a list of barcoded overhang adapter sequences that you can order from an oligo synthesis provider.
## REQUIRED MATERIALS & EQUIPMENT FOR MULTIPLEXED AMPLICON LIBRARY CONSTRUCTION USING BARCODED OVERHANG ADAPTERS

<table>
<thead>
<tr>
<th>ITEM</th>
<th>WHERE USED</th>
<th>VENDOR</th>
<th>PART NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barcoded Overhang Adapters</td>
<td>Library Prep</td>
<td>PacBio or Third-Party Oligo Vendor</td>
<td>See ‘Barcoded Overhang Adapters’ note below</td>
</tr>
<tr>
<td>SMRTbell Express Template Prep Kit 2.0</td>
<td>Library Prep</td>
<td>PacBio</td>
<td>100-938-900</td>
</tr>
<tr>
<td>AMPure PB Bead Kit</td>
<td>Purification</td>
<td>PacBio</td>
<td>100-265-900</td>
</tr>
<tr>
<td>8-Channel or 12-Channel Pipette</td>
<td>High-Throughput Pipetting</td>
<td>Any</td>
<td>Vendor-specific</td>
</tr>
<tr>
<td>PCR 8-Tube Strips</td>
<td>Tubes for reactions</td>
<td>Any</td>
<td>Vendor-specific</td>
</tr>
<tr>
<td>96-Well Plate</td>
<td>Tubes for reactions</td>
<td>Any</td>
<td>Vendor-specific</td>
</tr>
</tbody>
</table>

### Barcoded Overhang Adapters

- 16 Barcoded Overhang Adapters are commercially available from PacBio:
  - PacBio Barcoded Overhang Adapter Kit 8A (PN 101-628-400) and PacBio Barcoded Overhang Adapter Kit 8B (PN 101-628-500). We recommend using both sets for multiplexing up to 16 samples

- **If multiplexing projects require more than 16 samples**, PacBio provides a [list](#) of 96 barcoded overhang adapter (BOA) sequences that you can order from an oligo synthesis provider (see our [Multiplexing Resources](#) website)
  - List contains BOA sequence information (5’ → 3’) along with their recommended synthesis scale and purification method
  - Annealing procedure for forming SMRTbell hairpins is described in the “Annealing PacBio SMRTbell Overhang Adapters” section of this procedure
MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO BARCODED OVERHANG ADAPTERS: DETAILED WORKFLOW OVERVIEW

1. **Input PCR Amplicon QC**
   - Verify input amplicon sample concentration, purity and size distribution
   - Minimum required input mass per amplicon for library construction depends on amplicon size:
     - E.g., 10 ng for 150 bp amplicon to 150 ng for 15,000 bp amplicon

2. **Barcoded SMRTbell Express TPK 2.0 Library Construction**
   - Construct barcoded SMRTbell libraries using PacBio Barcoded Overhang Adapters
   - Typical library yield ≥40%
   - <5 hours hands-on time (depends on multiplex design)

3. **Sequencing Preparation**
   - Anneal Sequencing Primer, bind Polymerase, perform Complex Cleanup
   - For specific sample setup and run design guidance, refer to Loading and Pre-Extension Recommendations Quick Reference Cards for Sequel, Sequel II and Sequel IIe Systems

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1. PCR Product 1, 2, … n
2. DNA Damage Repair 1, 2, … n
3. End Repair/A-Tail 1, 2, … n
4. Ligation 1, 2, … n
5. Heat Kill Ligase
6. Sample Pooling
7. AMPure PB Bead Purification
8. Primer Annealing and Polymerase Binding
9. Sequencing
4. Data Analysis

- Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- De-multiplex barcodes within SMRT Link GUI or on the command line
- Amplicon data analysis using SMRT Analysis Applications (CCS, Minor Variants Analysis)
- Output data in standard file formats, (BAM and FASTA/Q) for seamless integration with downstream analysis tools

- PacBio highly recommends **SMRT Link v9.0 or higher** to perform demultiplexing of your amplicon sequencing data
- SMRT Link GUI supports demultiplexing of up to 10,000 barcoded samples per data set; support for >10,000 barcoded samples is available using the command-line interface
PACBIO BARCODED OVERHANG ADAPTER KIT 8A / 8B IS AVAILABLE FOR MULTIPLEXING UP TO 16 POOLED AMPLICON SAMPLES

- 16 Barcoded overhang adapters are commercially available from PacBio:
  - PacBio Barcoded Overhang Adapter Kit 8A, PN 101-628-400;
  - PacBio Barcoded Overhang Adapter Kit 8B, PN 101-628-500

- Each Barcoded Overhang Adapter (BOA) Kit 8A or 8B contains sufficient reagents to perform 12 ligation reactions for each BOA to support multiplexed amplicon sequencing

- Each PacBio barcode sequence is 16 bp in length

- We recommend using both BOA kits (8A and 8B) for multiplexing up to 16 samples

If multiplexing projects require >16 samples, PacBio’s Multiplexing Resources website provides a list of 96 barcoded overhang adapter (BOA) sequences that you can order from a third-party oligo synthesis provider

PacBio barcode sequence FASTA files for Sequel Systems can be obtained here: https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/
GENERAL RECOMMENDATIONS FOR PLANNING MULTIPLEXING EXPERIMENTS USING PACBIO BARCODED OVERHANG ADAPTERS

1. Review the section on Page 3 of the procedure that describes ‘Best Practices for Generating High-Quality PCR Products for PacBio Sequencing.’

2. Accurately assess the sizes of the amplicons that are being multiplexed. Pool amplicons of similar size (within +/- 15% of the mean size).

3. Determine the number of different amplicon samples to be multiplexed.
   - We recommend initially starting with a low number of amplicon samples to multiplex. As you become more experienced with the workflow and understand the sequencing coverage performance of your amplicons, you may decide to increase the level of multiplexing.

4. Estimate the input DNA mass requirement per amplicon for library construction.

5. Determine the appropriate concentration of AMPure PB beads to use for the purification steps.
   - See ‘Recommendations for AMPure PB Bead Purifications’ on Page 4 of the procedure.
ESTIMATING INPUT AMPLICON MASS REQUIREMENTS FOR MULTIPLEXED SMRTBELL LIBRARY CONSTRUCTION USING PACBIO BARCODED OVERHANG ADAPTERS

Refer to Table 2 on Page 3 of the procedure for DNA input requirements for SMRTbell library construction.

<table>
<thead>
<tr>
<th>AMPLICON SIZE (BP)</th>
<th>MINIMUM INPUT PER AMPLICON INTO DNA DAMAGE REPAIR (ng)</th>
<th>TARGET DNA MASS (ng) AFTER POOLING*</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 – 500</td>
<td>10 ng per amplicon for ≥25-plex</td>
<td>250</td>
</tr>
<tr>
<td>501 – 1000</td>
<td>10 ng per amplicon for ≥25-plex</td>
<td>250</td>
</tr>
<tr>
<td>1001 – 3000</td>
<td>50 ng per amplicon for ≥10-plex</td>
<td>500</td>
</tr>
<tr>
<td>3001 – 10000</td>
<td>100 ng per amplicon for ≥10-plex</td>
<td>1000</td>
</tr>
<tr>
<td>10001 - 15000</td>
<td>150 ng per amplicon for ≥10-plex</td>
<td>1500</td>
</tr>
</tbody>
</table>

* The value in the “Target DNA Mass (ng) After Pooling” column is the recommended minimum amount of SMRTbell library after pooling of BOA ligation products.

- The numbers in the “Minimum Input per Amplicon into DNA Damage Repair” column are the recommended per-amplicon masses required for the first enzymatic reaction step in library construction (DNA Damage Repair), regardless of the number of amplicons to be multiplexed.
After performing the ligase heat kill, pool all reactions (~16.3 µL each) into a single 1.5- or 2.0-mL DNA Lo-Bind tube.

2. Mix and spin down the contents of the tube with a quick spin in a microfuge.

3. Proceed to the next step to purify the pooled library with AMPure PB beads.

Ensure that the pooled sample contains the minimum required total mass (ng) of SMRTbell library as indicated in Table 2 ('Target DNA Mass (ng) After Pooling' column)

Heat kill of ligase prior to pooling is necessary in order to prevent unwanted further ligation of different combinations of residual barcoded overhang adapter hairpins to any open-ended SMRTbell templates present in the reaction tube.

Table 2

<table>
<thead>
<tr>
<th>Target DNA Mass (ng) After Pooling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum Required Mass (ng)</td>
</tr>
<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>1.5 µg</td>
</tr>
<tr>
<td>2.0 µg</td>
</tr>
</tbody>
</table>

Heat kill of ligase prior to pooling is necessary in order to prevent unwanted further ligation of different combinations of residual barcoded overhang adapter hairpins to any open-ended SMRTbell templates present in the reaction tube.
USE A MULTI-CHANNEL PIPETTE FOR HIGH-THROUGHPUT AMPLICON SEQUENCING APPLICATIONS

When working with a large number of reactions, PacBio highly recommends using a multichannel pipette to transfer small aliquots of master mixes to a 96-well PCR plate or PCR tubes.

1. Prepare master mixes according to the instructions in the DNA Damage Repair, End-Repair/A-tailing and Adapter Ligation sections of this procedure, or use the Express Amplicon Master Mix Calculator.

2. Transfer aliquots of the master mix into an 8-tube strip using a single channel pipette (1/8th master mix volume to each of the eight well of the strip tube). Each tube can accommodate up to 200 µL of liquid.

3. Using an 8-channel pipette, transfer the required reaction volume of the master mix from the 8-tube strip into the appropriate sample wells of a 96-well plate.

4. Repeat until all required reaction wells in the sample plate are filled.
Multiplexed Amplicon Library Preparation Using PacBio Barcoded Universal Primers (BUP)
PROCEDURE & CHECKLIST – PREPARING SMRTBELL LIBRARIES USING PACBIO BARCODED UNIVERSAL PRIMERS FOR MULTIPLEXING AMPICONS

- This protocol (PN 101-791-800) describes a 2-step PCR method for generating up to 96 symmetrically barcoded amplicon products using the PacBio Barcoded Universal Primers (BUP) kit (PN 101-629-100) and performing SMRTbell library construction for multiplexed BUP amplicon sequencing on Sequel, Sequel II and Sequel IIe Systems.

- Protocol document contains:
  1. Best practices recommendations for generating high-quality PCR products for PacBio sequencing
  2. Instructions for generating symmetrically barcoded amplicons using a 2-Step PCR method with PacBio’s Barcoded Universal Primer Kit* and performing equimolar pooling of barcoded amplicon products
  3. Instructions for constructing SMRTbell libraries with pooled barcoded PCR amplicons using PacBio’s SMRTbell Express Template Prep Kit 2.0
  4. Sample setup guidance for preparing multiplexed amplicon SMRTbell libraries for sequencing on the Sequel, Sequel and Sequel IIe Systems

- For guidance on generating asymmetrically barcoded amplicon samples for higher multiplexing (>96-plex) using customer-supplied barcoded M13 primers, see Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing (PN 101-921-300) and Appendix 1 of this presentation

- For guidance on preparing HLA amplicon samples for multiplexed SMRT Sequencing, see Reference Guide – Human Leukocyte Antigen (HLA) Sequencing (PN 102-066-200) and Appendix 2 of this presentation
# REQUIRED MATERIALS & EQUIPMENT FOR MULTIPLEXED AMPLICON LIBRARY CONSTRUCTION USING BARCODED UNIVERSAL PRIMERS

<table>
<thead>
<tr>
<th>ITEM</th>
<th>WHERE USED</th>
<th>VENDOR</th>
<th>PART NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target Specific Primers tailed with F/R Universal Sequences (Customer-supplied)</td>
<td>PCR Amplification (1st-Round)</td>
<td>Oligo Synthesis Company</td>
<td>N/A</td>
</tr>
<tr>
<td>Barcoded Universal F/R Primers Plate-96v2</td>
<td>PCR Amplification (2nd-Round)</td>
<td>PacBio</td>
<td>101-629-100</td>
</tr>
<tr>
<td>High Fidelity DNA Polymerase Master Mix (Two recommended mixes are listed below)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phusion High-Fidelity PCR Master Mix with HF Buffer; or</td>
<td>PCR Amplification</td>
<td>Thermo Scientific</td>
<td>F531S</td>
</tr>
<tr>
<td>KAPA HiFi HotStart ReadyMix PCR Kit</td>
<td>PCR Amplification</td>
<td>Roche</td>
<td>KK2600, KK2601, or KK2602</td>
</tr>
<tr>
<td>SMRTbell Express Template Prep Kit 2.0</td>
<td>Library Prep</td>
<td>PacBio</td>
<td>100-938-900</td>
</tr>
<tr>
<td>AMPure PB Bead Kit</td>
<td>Purification</td>
<td>PacBio</td>
<td>100-265-900</td>
</tr>
<tr>
<td>Multichannel Pipettor</td>
<td>High-Throughput Pipetting</td>
<td>Any</td>
<td>Vendor-specific</td>
</tr>
</tbody>
</table>
MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO BARCODED UNIVERSAL PRIMERS: DETAILED WORKFLOW OVERVIEW

1. 2-Step PCR Amplicon Generation with Barcoded Universal Primer Kit (PN 101-629-100)
   - Perform 1st-round PCR using Target Specific Primers tailed with Universal Sequences (US)
   - Perform 2nd-round PCR Using Barcoded Universal Primers (BUP)
   - Perform equimolar pooling of barcoded amplicon products
   - Purify single, pooled amplicon sample using AMPure PB beads

2. SMRTbell Express TPK 2.0 Library Construction
   - Required input mass per pooled sample for library construction is ≥250 ng (depending on the insert size)
   - Typical library yield ≥40%
   - ~4 hours hands-on time
3. Sequencing Preparation
- Anneal Sequencing Primer, bind Polymerase, perform Complex Cleanup
- For specific sample setup and run design guidance, refer to Loading and Pre-Extension Recommendations Quick Reference Cards for Sequel, Sequel and Sequel IIe Systems

4. Data Analysis
- Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- De-multiplex barcodes within SMRT Link GUI or on the command line
- Amplicon data analysis using SMRT Analysis Applications (CCS, Minor Variants Analysis)
- Output data in standard file formats, (BAM and FASTA/Q) for seamless integration with downstream analysis tools

- PacBio highly recommends **SMRT Link v9.0 or higher** to perform demultiplexing of your amplicon sequencing data
- SMRT Link GUI supports demultiplexing of up to 10,000 barcoded samples per data set; support for >10,000 barcoded samples is available using the command-line interface
DESIGNING TARGET-SPECIFIC PRIMERS TAILED WITH UNIVERSAL SEQUENCES FOR 1ST-ROUND PCR AMPLIFICATION

PacBio highly recommends complying with the following requirements below when ordering oligos from your oligo synthesis provider

1. The 5’ end of the primer must be blocked (e.g., 5AmMC6) to prevent amplicons carried over from the first round of PCR from forming SMRTbell templates during library construction, which can reduce the yield of barcoded reads during sequencing.

2. Oligos can be desalted.

3. Use the following primer format:

<table>
<thead>
<tr>
<th>PRIMER TYPE</th>
<th>UNIVERSAL SEQUENCE</th>
<th>TARGET-SPECIFIC PRIMER</th>
<th>PRIMER TO ORDER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward_Internal_PCR_Primer</td>
<td>/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac</td>
<td>FOR EXAMPLE1</td>
<td>/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac FOR EXAMPLE1</td>
</tr>
<tr>
<td>Reverse_Internal_PCR_Primer</td>
<td>/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag</td>
<td>REV EXAMPLE2</td>
<td>/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag REV EXAMPLE2</td>
</tr>
</tbody>
</table>

The procedure was optimized using high fidelity Phusion Hot Start II for PCR. KAPA HiFi HotStart ReadyMix may also be used. PCR optimizations are highly recommended before proceeding with the second-round PCR step.
PACBIO BARCODED UNIVERSAL PRIMERS PLATE-96 KIT FOR 2ND-ROUND PCR AMPLIFICATION TO GENERATE SYMMETRICALLY BARCODED SAMPLES

- To perform this procedure, barcoded universal primers are required
- 96 barcoded universal (forward/reverse) primer pairs are commercially available from PacBio in a ready-to-use plate kit format:
  - Barcoded Universal F/R Primers Plate-96 Kit v2, PN 101-629-100
- Each well on the plate contains a primer pair consisting of forward and reverse primers having the same (16-bp) PacBio barcode sequence
- Each barcode is present in only one well for a total of 96 different barcodes on the plate. Each primer has a Universal Sequence tag on the 3' end.

Reagent kit quantities support a single use of each of the 96 primers pairs in the plate for SMRTbell library preparations.

PacBio barcode sequence FASTA files for Sequel Systems can be obtained here: https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/
GENERAL RECOMMENDATIONS FOR PLANNING MULTIPLEXING EXPERIMENTS USING PACBIO BARCODED UNIVERSAL PRIMERS

1. Review the section on Page 3 of the procedure that describes ‘Best Practices for Generating High-Quality PCR Products for PacBio Sequencing.’

2. Accurately assess the sizes of the amplicons that are being multiplexed.
   - Pool amplicons of similar size (within +/- 15% of the mean size).

3. Determine the number of different amplicon samples to be multiplexed.
   - We recommend initially starting with a low number of amplicon samples to multiplex. As you become more experienced with the workflow and understand the sequencing coverage performance of your amplicons, you may decide to increase the level of multiplexing.

4. Estimate the input DNA mass requirements per pooled amplicon sample for library construction. See ‘DNA Input Requirements for Library Construction’ section on Page 5 of the procedure.

5. Determine the appropriate concentration of AMPure PB beads to use for the purification steps. See ‘Recommendations for AMPure PB bead Purifications’ section on Page 5 of the procedure.
ESTIMATING INPUT DNA MASS REQUIREMENTS FOR MULTIPLEXED AMPLICON LIBRARY CONSTRUCTION USING PACBIO BARCODED UNIVERSAL PRIMERS

Refer to Table 4 on Page 5 of the procedure for DNA input requirements for library construction

<table>
<thead>
<tr>
<th>AMPLICON SIZE</th>
<th>REQUIRED INPUT DNA MASS PER AMPLICON POOL (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250-500 bp</td>
<td>250 – 500*</td>
</tr>
<tr>
<td>500-1000 bp</td>
<td>250 – 500*</td>
</tr>
<tr>
<td>1-3 kb</td>
<td>500 - 1000</td>
</tr>
<tr>
<td>3-10 kb</td>
<td>1000 - 2000</td>
</tr>
<tr>
<td>15 kb</td>
<td>1500 - 3000</td>
</tr>
</tbody>
</table>

* If input DNA mass is greater than 500 ng for amplicon sizes ≤1 kb, use 2X Barcoded Overhang Adapter in the ligation reaction.

- Note that the input requirements shown in Table 4 refer to the total pooled mass of DNA, not the mass of individual members of the pool. Samples should be present at equimolar concentrations after pooling (see “Best Practices for Equimolar Pooling” on Page 6 of the procedure).

- If necessary, replicate PCR reactions should be set up to obtain the required amount of DNA product.
  - This approach also minimizes PCR sampling bias for samples containing heterogeneous templates.
BEST PRACTICES FOR EQUIMOLAR POOLING OF BUP AMPICON SAMPLES FOR SMRTBELL LIBRARY CONSTRUCTION

For studies targeting a single consensus sequence per sample, amplicons may be multiplexed to leverage the throughput capacity of a single SMRT Cell.

1. Pooling is generally recommended for amplicons of similar sizes (within +/-15% of the mean size).

2. Ideally, amplicons should be AMPure PB bead purified prior to multiplexing. See “Concentrate PCR Products Using AMPure PB Beads” section of the procedure.

3. To obtain equal representation of each amplicon in the data, it is important to pool samples in equimolar amounts.
   - To do this, purify with AMPure PB beads and quantify each amplicon using an appropriate DNA sizing QC analysis tool (e.g., Agilent Bioanalyzer System, Agilent Fragment Analyzer System, or Agilent Femto Pulse System, etc.)
BEST PRACTICES FOR EQUIMOLAR POOLING OF BUP AMPLICON SAMPLES FOR SMRTBELL LIBRARY CONSTRUCTION

For studies targeting a single consensus sequence per sample, amplicons may be multiplexed to leverage the throughput capacity of a single SMRT Cell.

4. Remove non-specific PCR products (containing bands) prior to pooling. The presence of non-specific products in the pool will impact sequencing data yield.
   a. If amplicons contain secondary bands that are <1.5 kb, it may be possible to remove them from >3 kb amplicons using AMPure PB bead purification using an appropriate concentration of beads.
   b. If the contaminating bands are close in size to or are larger than the desired amplicon, or are greater than 1.5 kb (i.e., they cannot be removed by AMPure PB bead purification), size selection using an automated size selection tool or other gel-based method may be necessary.
   c. If removal of contaminating bands is not possible, we recommend re-optimization of the amplification reaction using more stringent PCR conditions.
   d. Always determine the concentration of the amplicon target band or peak only, and use this value to the mass or volume of the amplicon sample to be used during pooling.
   e. If presence of contaminating bands is determined to be acceptable, you may choose to include the amplicon in the sample pool. In such cases, it may be necessary to increase the relative input amounts of such amplicons during pooling in order to achieve adequate sequencing data yields.
   f. Always determine the concentration of the target band or peak.
5. For higher multiplexing (>96-plex), purifying and quantifying each individual PCR product may be difficult or impractical. A QC method that may work well is to load samples on an agarose gel to view the PCR products prior to pooling. This QC method may work well if PCR conditions are fully optimized to generate clean specific PCR products consistently.

   a. PCR products that show the same band intensity on a gel may be pooled by volume or mass.
      - To do this, include control fragments of known concentrations when loading samples to perform agarose gel electrophoresis. The pooled samples must meet the minimum input requirements listed on Table 4.

   b. For samples that show weak signals on a gel, increase the volume or mass used during pooling.
USING A MULTI-CHANNEL PIPETTE FOR HIGH-THROUGHPUT AMPLICON SEQUENCING APPLICATIONS

When working with a large number of reactions, PacBio highly recommend using a multi-channel pipette to transfer small aliquots of master mixes to a 96-well PCR plate or PCR tubes

1. Prepare master mixes according to the instructions in the ‘First-Round PCR Using Target Specific Primers Tailed with a Universal Sequence’ and ‘A Second-Round PCR Using Barcoded Universal Primers’ sections of this procedure.

2. Transfer aliquots of the master mix into an 8-tube strip using a single channel pipette (1/8th master mix volume to each of the eight well of the strip tube). Each tube can accommodate up to 200 µL of liquid.

3. Using an 8-channel pipette, transfer the required reaction volume of the master mix from the 8-tube strip into the appropriate sample wells of a 96-well plate.

4. Repeat until all required reaction wells in the sample plate are filled.
Multiplexed Amplicon Library Sequencing Workflow Recommendations
SAMPLE SETUP RECOMMENDATIONS FOR AMPLICON LIBRARIES – SEQUEL SYSTEM (CHEMISTRY 3.0)

Sequel System Sample Setup Procedure for Amplicons ≥3 kb and <3 kb

- Follow SMRT Link Sample Setup instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel System for sequencing amplicon samples ≥3 kb and <3 kb

- Anticipated recovery yield after complex cleanup with AMPure PB beads*:
  - ≥5 – 6 kb Amplicon Libraries: ~50%
  - ~3 – 4 kb Amplicon Libraries: ~40%
  - ~1 – 2 kb Amplicon Libraries: ~30%

* ProNex beads can also be used for complex cleanup and may lead to improved library recovery efficiencies for short (<3 kb) amplicon samples. To help improve accuracy of DNA quantitation of short amplicon samples during complex cleanup, use >1 µL of sample volume for Qubit assays.

Loading and Pre-Extension Time Recommendations for the Sequel® System

<table>
<thead>
<tr>
<th>Applications</th>
<th>Pre-Extension Time (hr)</th>
<th>Movie Collection Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicons (≥3kb)</td>
<td>Use default values in Run Design</td>
<td>6 - 20</td>
</tr>
<tr>
<td>Amplicons (&lt;3kb)</td>
<td>Use default values in Run Design</td>
<td>6 - 20</td>
</tr>
</tbody>
</table>
SAMPLE SETUP RECOMMENDATIONS FOR AMPLICON LIBRARIES – SEQUEL II AND Ile SYSTEMS (CHEMISTRY 2.0)

Sequel II and Ile System Sample Setup Procedure for Amplicons ≥3 kb

- Follow SMRT Link Sample Setup instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/Ile Systems for preparing ≥3 kb amplicon samples for sequencing

- For SMRT Link v10.0 (or higher): Select ‘≥3 kb Amplicon Sequencing’ from the Application field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface

<table>
<thead>
<tr>
<th>Applications</th>
<th>Data Type</th>
<th>Library Prep Kit</th>
<th>Binding Kit</th>
<th>Sequencing Primer</th>
<th>Pol Binding Time (hr)</th>
<th>Complex Cleanup</th>
<th>Loading Concentration Range (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicons (≥3 kb)</td>
<td>CCS</td>
<td>Express Prep 2.0</td>
<td>Binding Kit 2.0</td>
<td>v4</td>
<td>1</td>
<td>1.2X AMPure PB Beads</td>
<td>30 - 70</td>
</tr>
<tr>
<td>Amplicons (&lt; 3 kb)</td>
<td>CCS</td>
<td>Express Prep 2.0</td>
<td>Binding Kit 2.1</td>
<td>v4</td>
<td>1</td>
<td>1.2X AMPure PB Beads</td>
<td>40 - 150</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Applications</th>
<th>Pre-Extension Time (hr)</th>
<th>Movie Collection Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicons (≥3 kb)</td>
<td>Use default values in Run Design</td>
<td>10 - 30</td>
</tr>
<tr>
<td>Amplicons (&lt; 3 kb)</td>
<td>Use default values in Run Design</td>
<td>10</td>
</tr>
</tbody>
</table>

- Anticipated recovery yield after complex cleanup with AMPure PB beads*:
  - ≥5 – 6 kb Amplicon Libraries: ~50%
  - ~3 – 4 kb Amplicon Libraries: ~40%

* ProNex beads can also be used for complex cleanup and may lead to improved library recovery efficiencies for short (<3 kb) amplicon samples. To help improve accuracy of DNA quantitation of short amplicon samples during complex cleanup, use >1 µL of sample volume for Qubit assays.
SAMPLE SETUP RECOMMENDATIONS FOR AMPLICON LIBRARIES – SEQUEL II AND Ile SYSTEMS (CHEMISTRY 2.0) (CONT.)

Sequel II and Ile System Sample Setup Procedure for Amplicons <3 kb

- Follow SMRT Link Sample Setup instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/Ile Systems for preparing <3 kb amplicon samples for sequencing

- For SMRT Link v10.0 (or higher): Select ‘<3 kb Amplicon Sequencing’ from the Application field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface

- Anticipated recovery yield after complex cleanup with AMPure PB beads*:
  - ~3 kb Amplicon Libraries: ~40%
  - ~1 – 2 kb Amplicon Libraries: ~30%

* ProNex beads can also be used for complex cleanup and may lead to improved library recovery efficiencies for short (<3 kb) amplicon samples. To help improve accuracy of DNA quantitation of short amplicon samples during complex cleanup, use >1 µL of sample volume for Qubit assays.
Multiplexed Amplicon Data Analysis Recommendations
DATA ANALYSIS SOLUTIONS FOR MULTIPLEXED SEQUENCING OF SMRTBELL EXPRESS TPK 2.0 AMPLICON LIBRARIES

Use SMRT Link and other analysis tools to fully characterize genetic complexity – structural variation, rare SNPs, indels, CNV, microsatellites, haplotypes, and phasing

- Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System

- ≥50-fold HiFi read coverage per target locus is recommended for variant detection applications

- 6,000-fold HiFi read coverage per target locus is recommended for minor variant detection (1% sensitivity) applications

- PacBio highly recommends upgrading to SMRT Link v9.0 or higher to perform de-multiplexing of your amplicon sequencing data
  - SMRT Link supports demultiplexing up to 10,000 barcoded samples per data set
  - Refer to Barcoding Overview documents available on PacBio’s Multiplexing Resources website (https://www.pacb.com/products-and-services/analytical-software/multiplexing/) for detailed information on QC metrics for evaluation of barcoding performance using SMRT Link
    - SMRT Analysis Barcoding Overview (v9.0) (PN 101-923-200)

- Can use SMRT Link to detect, quantitate, and phase single nucleotide polymorphisms within coding regions using the Minor Variants Analysis (MVA) application

- Output data in standard file formats, (BAM and FASTA/Q) for seamless integration with downstream analysis tools

- HiFi reads are compatible with standard analysis tools for variant calling such as Google DeepVariant
HIFI (Q20, Q30 CCS) READ YIELD PERFORMANCE FOR DIFFERENT LIBRARY INSERT SIZES (SEQUEL II CHEMISTRY 2.0)

### HIFI READ YIELD (50% P1)

<table>
<thead>
<tr>
<th>INSERT SIZE</th>
<th>MOVIE LENGTH</th>
<th>ESTIMATED ≥Q20 HIFI READS</th>
<th>ESTIMATED ≥Q30 HIFI READS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 kb</td>
<td>10 hours</td>
<td>3,000,000</td>
<td>2,500,000</td>
</tr>
<tr>
<td>5 kb</td>
<td>10 hours</td>
<td>2,000,000</td>
<td>1,750,000</td>
</tr>
<tr>
<td>5 kb</td>
<td>20 hours</td>
<td>2,000,000</td>
<td>1,850,000</td>
</tr>
<tr>
<td>12 kb</td>
<td>30 hours</td>
<td>1,750,000</td>
<td>1,250,000</td>
</tr>
<tr>
<td>16 kb</td>
<td>30 hours</td>
<td>1,500,000</td>
<td>1,000,000</td>
</tr>
<tr>
<td>20 kb</td>
<td>30 hours</td>
<td>1,250,000</td>
<td>500,000</td>
</tr>
</tbody>
</table>

Data shown above are from DNA libraries constructed using the SMRTbell Express Template Prep Kit 2.0 and sequenced on a Sequel II System (Chemistry 2.0) using different movie collection times. Read lengths, reads/data per SMRT Cell 8M and other sequencing performance results vary based on sample quality/type and insert size.
Multiplexed Amplicon Library Example Sequencing Performance Data
EXAMPLE 1: BARCODED OVERHANG ADAPTER (BOA) MULTIPLEXED AMPLICON SEQUENCING PERFORMANCE

96-plex Human Amplicon Library Prepared Using PacBio-Barcoded Overhang Adapters

A. Example Primary Sequencing Performance Metrics (Sequel II System)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TOTAL BASES (Gb)</th>
<th>UNIQUE MOLECULAR YIELD (Gb)</th>
<th>MEAN POLYMERASE RL (bp)</th>
<th>POLYMERASE RL N50 (bp)</th>
<th>MEAN LONGEST SUBREAD (bp)</th>
<th>LONGEST SUBREAD N50 (bp)</th>
<th>P0 (%)</th>
<th>P1 (%)</th>
<th>P2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-Plex Human Amplicon Library (~3.1 kb – 5.7 kb)</td>
<td>276.8</td>
<td>35.4</td>
<td>52853</td>
<td>121339</td>
<td>7828</td>
<td>15667</td>
<td>32.0</td>
<td>65.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Sequel II System Chemistry 2.0; 60 pM on-plate loading concentration; 20-hour movie collection time; 1.4-hour pre-extension time

- 16 human gDNA samples were taken into PCR amplification and all 16 were symmetrically-barcoded with 6 unique barcodes per sample to generate a 96-plex pooled SMRTbell library
  - Measured amplicon sizes for each barcoded sample ranged from ~3.1 kb to 5.7 kb
- 96-plex pooled amplicon library was sequenced on a single Sequel II SMRT Cell 8M using a 20-h movie collection time

* Read lengths, reads/data per SMRT Cell and other sequencing performance results vary based on sample quality/type and insert size.
EXAMPLE 1: BARCODED OVERHANG ADAPTER (BOA) MULTIPLEXED AMPLICON SEQUENCING PERFORMANCE (CONT.)

96-plex Human Amplicon Library Prepared Using PacBio-Barcoded Overhang Adapters

B. Example CCS and Barcode Demultiplexing Analysis Performance Metrics

- ~2.4 million HiFi reads (45%) were obtained from ~5.2 million P1 polymerase reads generated on a single Sequel II SMRT Cell 8M
- ~2.3 million barcoded reads (97%) were recovered after performing demultiplexing analysis of ~2.4 million input HiFi reads using SMRT Link

<table>
<thead>
<tr>
<th>CCS ANALYSIS SUMMARY METRICS</th>
<th>VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiFi (≥Q20 CCS) Reads</td>
<td>2,370,623</td>
</tr>
<tr>
<td>HiFi Base Yield (bp)</td>
<td>8,725,080,599</td>
</tr>
<tr>
<td>HiFi Read Length (mean, bp)</td>
<td>3,680</td>
</tr>
<tr>
<td>HiFi Read Quality (median)</td>
<td>Q40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DEMULTIPLEXING ANALYSIS SUMMARY METRICS</th>
<th>VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiFi (≥Q20 CCS) Reads Input for Demultiplexing Analysis</td>
<td>2,370,623</td>
</tr>
<tr>
<td>Unique Barcodes Identified</td>
<td>96</td>
</tr>
<tr>
<td>Barcoded HiFi Reads</td>
<td>2,288,576</td>
</tr>
<tr>
<td>Unbarcoded Reads</td>
<td>82,047</td>
</tr>
<tr>
<td>Mean HiFi Reads per Barcode</td>
<td>23,839</td>
</tr>
<tr>
<td>Max. HiFi Reads per Barcode</td>
<td>38,905</td>
</tr>
<tr>
<td>Min. HiFi Reads per Barcode</td>
<td>12,469</td>
</tr>
<tr>
<td>Mean HiFi Read Length (bp) of Barcoded Reads</td>
<td>3,671</td>
</tr>
</tbody>
</table>

Sequel II System Chemistry 2.0; 60 pM on-plate loading concentration; 20-hour movie collection time; 1.4-hour pre-extension time; SMRT Link v9.0.
EXAMPLE 2: BARCODED UNIVERSAL PRIMER (BUP) MULTIPLEXED AMPLICON SEQUENCING PERFORMANCE

92-plex Bacterial Amplicon Library Prepared Using PacBio-Barcoded M13 Universal Primers

A. Example Primary Sequencing Performance Metrics (Sequel II System)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TOTAL BASES (Gb)</th>
<th>UNIQUE MOLECULAR YIELD (Gb)</th>
<th>MEAN POLYMERASE RL (bp)</th>
<th>POLYMERASE RL N50 (bp)</th>
<th>MEAN LONGEST SUBREAD (bp)</th>
<th>LONGEST SUBREAD N50 (bp)</th>
<th>P0 (%)</th>
<th>P1 (%)</th>
<th>P2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>92-Plex Bacterial Amplicon Library (~2.6 kb)</td>
<td>128.7</td>
<td>14.9</td>
<td>23662</td>
<td>56425</td>
<td>3086</td>
<td>2733</td>
<td>27.0</td>
<td>67.9</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Sequel II System Chemistry 2.0; 50 pM on-plate loading concentration; 10-hour movie collection time; 0.6-hour pre-extension time

- Amplicons were generated using *E. coli* gDNA
  - Measured amplicon size is ~2.6 kb
- Replicate amplicon samples were asymmetrically-barcoded with 96 unique barcoded M13 universal primer pairs to generate a 92-plex pooled SMRTbell library
- 92-plex pooled bacterial amplicon library was sequenced on a single Sequel II SMRT Cell 8M using a 10-h movie collection time

*Read lengths, reads/data per SMRT Cell and other sequencing performance results vary based on sample quality/type and insert size.*
**EXAMPLE 2: BARCODED UNIVERSAL PRIMER (BUP) MULTIPLEXED AMPLICON SEQUENCING PERFORMANCE (CONT.)**

92-plex Bacterial Amplicon Library Prepared Using PacBio-Barcoded M13 Universal Primers

**B. Example CCS and Barcode Demultiplexing Analysis Performance Metrics**

**CCS ANALYSIS SUMMARY METRICS**

<table>
<thead>
<tr>
<th>ANALYSIS METRIC</th>
<th>VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiFi (≥Q20 CCS) Reads</td>
<td>2,435,305</td>
</tr>
<tr>
<td>HiFi Base Yield (bp)</td>
<td>5,513,188,488</td>
</tr>
<tr>
<td>HiFi Read Length (mean, bp)</td>
<td>2,263</td>
</tr>
<tr>
<td>HiFi Read Quality (median)</td>
<td>Q40</td>
</tr>
</tbody>
</table>

- ~2.4 million HiFi reads (45%) were obtained from ~5.4 million P1 polymerase reads generated on a single Sequel II SMRT Cell 8M
- ~2.3 million barcoded reads (95%) were recovered after performing demultiplexing analysis of ~2.4 million input HiFi reads using SMRT Link

**DEMULTIPLEXING ANALYSIS SUMMARY METRICS**

<table>
<thead>
<tr>
<th>ANALYSIS METRIC</th>
<th>VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiFi (≥Q20 CCS) Reads Input for Demultiplexing Analysis</td>
<td>2,435,305</td>
</tr>
<tr>
<td>Unique Barcodes Identified</td>
<td>92</td>
</tr>
<tr>
<td>Barcoded HiFi Reads</td>
<td>2,311,437</td>
</tr>
<tr>
<td>Unbarcoded Reads</td>
<td>123,868</td>
</tr>
<tr>
<td>Mean HiFi Reads per Barcode</td>
<td>25,124</td>
</tr>
<tr>
<td>Max. HiFi Reads per Barcode</td>
<td>50,999</td>
</tr>
<tr>
<td>Min. HiFi Reads per Barcode</td>
<td>22,429</td>
</tr>
<tr>
<td>Mean HiFi Read Length (bp) of Barcoded Reads</td>
<td>2,243</td>
</tr>
</tbody>
</table>

Sequel II System Chemistry 2.0; 50 pM on-plate loading concentration; 10-hour movie collection time; 0.6-hour pre-extension time; SMRT Link v9.0.
BEST PRACTICES: TARGETED SEQUENCING FOR AMPLICONS

Template Preparation with SMRTbell Express Template Prep Kit 2.0
- Start with high-quality, double-stranded DNA for PCR amplification
- Create SMRTbell templates between ~250 bp to 20 kb from ≥10 ng input mass per amplicon
- Optimize data throughput with flexible barcoding options:
  - Amplify PCR products using target-specific primers with incorporated barcodes; OR
  - Attach Barcoded Overhang Adapters during ligation without modifying existing primers; OR
  - Add Barcoded Universal Primers into amplicons via a simple 2-step PCR process

Sequence on the Sequel, Sequel II or Sequel IIe Systems
- Maximize output and turn-around-time with adjustable run parameters
  - For inserts <5 kb, recommend 10-hour movie collection time; for inserts ≥5 kb, recommend 20-hour or longer movie collection time
  - Generate up to ~3 million HiFi reads per Sequel II System SMRT Cell 8M*
- Sequence to desired coverage based on project needs
  - ≥50-fold coverage of HiFi reads per target locus is recommended for variant detection
  - 6,000-fold HiFi read coverage per target locus is recommended for minor variant detection (1% sensitivity)

Data Analysis Solutions with the PacBio Analytical Portfolio
- Fully characterize genetic complexity – structural variation, rare SNPs, indels, CNV, microsatellites, haplotypes, and phasing
- Utilize a variety of analysis tools within SMRT Link
  - Generate highly accurate HiFi single-molecule reads using Circular Consensus Sequencing (CCS)
  - Detect, quantitate, and phase single nucleotide polymorphisms within coding regions using Minor Variants Analysis
  - Easily de-multiplex barcodes within SMRT Link
- Output data in standard file formats for integration with downstream analysis tools
- HiFi long reads are compatible with standard analysis tools for variant calling such as Google DeepVariant

* Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.
TECHNICAL DOCUMENTATION & APPLICATIONS SUPPORT RESOURCES FOR MULTIPLEXED AMPLICON LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS

Sample Preparation Literature

- Application Brief: Targeted Sequencing for Amplicons – Best Practices (PN BP105-071919)
- Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplex SMRT Sequencing (PN 101-791-700)
- Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplex SMRT Sequencing (PN 101-791-800)
- Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing (PN 101-921-300)
- Reference Guide – Human Leukocyte Antigen (HLA) Sequencing (PN 102-066-200)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel System (PN 101-461-600)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel II System (PN 101-769-100)
- Overview – Sequel Systems Application Options and Sequencing Recommendations (PN 101-851-300)
- Application Consumable Bundles Purchasing Guide (PN PG100-051320)
- Technical Note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (PN TN101-061920)
- Technical Overview: Multiplexed Amplicon Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-814-300)
Data Analysis Resources

- **SMRT Analysis Barcoding Overview (v9.0)** (PN 101-923-200)
  - Contains detailed information on barcoding experimental design options and describes QC metrics for evaluation of barcoding performance using SMRT Link

- **PacBio Multiplexing Resources** Website: [https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/](https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/)
  - Barcoding Overview documents for different SMRT Link software versions
  - PacBio barcode sequence files (compressed FASTA) for use with Sequel, Sequel II and Sequel Ile Systems
  - Barcoded oligo ordering sheets

Example PacBio Data Sets

<table>
<thead>
<tr>
<th>TARGETED SEQUENCING APPLICATION</th>
<th>DATASET</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA Sequencing</td>
<td>Analysis of HLA Amplicons (HLA-(A), -(B), -(C), -(DRB1), -(DQB1) and –(DPB1)) Generated Using GenDX NGSgo-MX6-1 Kit</td>
</tr>
<tr>
<td>Viral Sequencing</td>
<td>SARS-CoV-2 – ATCC Control with Eden Primers</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DATA TYPE</th>
<th>PACBIO SYSTEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiFi Reads</td>
<td>Sequel II System</td>
</tr>
<tr>
<td>HiFi Reads</td>
<td>Sequel II System</td>
</tr>
</tbody>
</table>
TECHNICAL DOCUMENTATION & APPLICATIONS SUPPORT RESOURCES FOR MULTIPLEXED AMPLICON LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS (CONT.)

Posters

- ASHG 2019 Poster: The value of long read amplicon sequencing for clinical applications.
- ASHG 2019 Poster: TLA & long-read sequencing: Efficient targeted sequencing and phasing of the CFTR gene
- ASHG 2018 Poster: A simple segue from Sanger to high-throughput SMRT Sequencing with a M13 barcoding system
- ESHG 2018 Poster: High-throughput SMRT Sequencing of clinically relevant targets

Publications

Appendix 1: Multiplexed Amplicon Library Preparation Using PacBio Barcoded M13 Primers
PROCEDURE & CHECKLIST – PREPARING SMRTBELL LIBRARIES USING PACBIO BARCODED M13 PRIMERS FOR MULTIPLEX SMRT SEQUENCING

- This protocol (PN 101-921-300) describes a 2-step PCR method for generating up to 1,024 asymmetrically barcoded amplicon products using barcoded M13 primers and performing SMRTbell library construction for multiplexed amplicon sequencing on Sequel, Sequel II and Sequel IIe Systems (Sequel Systems)

- Protocol document contains:
  1. Best practices recommendations for generating high-quality PCR products for PacBio sequencing
  2. Instructions for generating asymmetrically barcoded amplicons using a 2-Step PCR method with customer-supplied (forward & reverse) barcoded M13 primers and performing equimolar pooling of barcoded amplicon products
  3. Instructions for constructing SMRTbell libraries with pooled barcoded PCR amplicons using PacBio’s SMRTbell Express Template Prep Kit 2.0
  4. Sample setup guidance for preparing multiplexed amplicon SMRTbell libraries for sequencing on the Sequel Systems

- This procedure supports amplicon sizes up to 10 kb.
  - Note that for amplicons >10 kb, two rounds of PCR are not recommended especially for targets with difficult or problematic sequence contexts, such as repeat expansions.
Appendix 2: Sample Preparation Recommendations for HLA Amplicon SMRT Sequencing
REFERENCE GUIDE – HUMAN LEUKOCYTE ANTIGEN (HLA) SEQUENCING

- This reference document (PN 102-066-200) describes recommended procedures for sequencing HLA Class I and Class II genes on the Sequel, Sequel II and Sequel Ile Systems for producing fully-phased, unambiguous, allele-level information.

- Reference guide contains:
  1. Best practices recommendations for input genomic DNA QC and generating high-quality PCR products for PacBio sequencing using either commercial PCR primers (GenDx HLA Typing Kit) or customer-designed PCR primers.
  2. Recommendations for planning multiplexing experiments using Barcoded Overhang Adapters (PN 101-791-700), Barcoded Universal Primers (PN 101-791-800), or Barcoded M13 Primers (PN 101-921-300).
  3. Workflow summary overview for constructing barcoded HLA SMRTbell libraries with input PCR amplicons using PacBio’s SMRTbell Express Template Prep Kit 2.0.
  4. Sample setup guidance for preparing multiplexed HLA amplicon SMRTbell libraries for sequencing on the Sequel, Sequel II and Sequel Ile Systems.

- If you are using a commercial GenDx HLA Typing Kit to prepare HLA amplicons for analysis, refer to Part A of this Reference Guide for recommended procedures for constructing a multiplexed SMRTbell library for SMRT Sequencing.

- If using your own custom-designed PCR primers to amplify HLA genes for analysis, proceed directly to Part B of this Reference Guide for sample preparation guidance.

1 Barcoded Overhang Adapter Kits 8A & 8B support multiplexing of up to 16 samples. If multiplexing projects require more than 16 samples (up to 96 samples), we offer a list of barcoded overhang adapter sequences that you can order from an oligo synthesis provider.

2 PacBio Barcoded Universal Primers kit (PN 101-629-100) supports generating up to 96 symmetrically barcoded amplicon product.

3 Barcoded M13 Primers protocol supports generating up to 1,024 asymmetrically barcoded amplicon products using barcoded M13 primers.

https://www.pacb.com/support/documentation/
GenDx (gendx.com) offers validated HLA primers with ready-to-use PCR master mixes, protocols and tools for SMRT Sequencing on Sequel, Sequel II and Sequel IIe Systems

SMRTBELL LIBRARY PREPARATION WORKFLOW OVERVIEW FOR HLA AMPLICON SAMPLES GENERATED WITH GenDX HLA TYPING KITS

- Depending on your project requirements, 3 kits are available from GenDX for typing up to 11 HLA loci
- PacBio has validated the GenDx NGSgo-MX6-1 Kit
  - Features six HLA loci in one tube for the amplification of HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 (3 – 6 kb target amplicon sizes)
  - Kit consists of 3 reagent tubes supporting up to 96 PCR reactions total:
    - NGSgo-MX6-1 primer mix
    - GenDx-LongMix PCR master mix (4x)
    - Nuclease-free water
- To reduce cost per sample, multiple samples may be barcoded using Barcoded Overhang Adapters and pooled for sequencing on a single SMRT Cell
  - Up to 48 barcoded samples may be pooled for sequencing on the Sequel System and up to 96 barcoded samples may be pooled for sequencing on the Sequel II and IIe Systems
  - Refer to Procedure & Checklist - Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplexing Amplicons (PN 101-791-700) for barcoding protocol details
- HLA typing analysis of HiFi reads can be performed with GenDx NGSengine software
SMRTBELL LIBRARY PREPARATION WORKFLOW OVERVIEW FOR HLA AMPLICON SAMPLES GENERATED WITH CUSTOMER-DESIGNED ASSAYS

HLA amplicon samples generated with **customer-designed assays** can be constructed to SMRTbell libraries and sequenced on Sequel, Sequel II and Sequel Ile Systems

- **Genomic DNA QC**
- **Multiplex PCR Using Customer-Designed Primers** and SMRTbell Library Construction Using Barcoded Overhang Adapters, Barcoded Universal Primers or Barcoded M13 Primers (Min. 150 ng of PCR product required per sample)
- **Pool Barcoded Libraries**
- **AMPure PB Bead Purification**
- **SMRT Sequencing**
- **Data Analysis Using GenDX NGSengine HLA Typing Software**

### Workflow for amplification and sequencing of HLA amplicons generated with customer-designed PCR primers. For procedure details, refer to **Reference Guide – HLA Sequencing (PN 102-066-200)**.

- **NOTE:** Users may design their own custom primers to amplify HLA gene targets for SMRT Sequencing – but be aware that painstaking and time-consuming validation experiments may be required to develop an optimized sample preparation workflow.

- **We therefore highly recommend using validated and ready-to-use primers from GenDx to amplify HLA gene targets for PacBio sequencing** (See Part A of **Reference Guide – HLA Sequencing (PN 102-066-200)**).

- To reduce cost per sample, multiple samples may be barcoded and pooled for sequencing on a single SMRT Cell
  - Up to 48 barcoded samples may be pooled for sequencing on the Sequel System and up to 96 barcoded samples may be pooled for sequencing on the Sequel II and Ile Systems
  - Three barcoding strategies are available; see Procedure & Checklist protocols for **Barcoded Overhang Adapters (PN 101-791-700)**, Barcoded Universal Primers (PN 101-791-800) or **Barcoded M13 Primers (PN 101-921-300)**

- HLA typing analysis of HiFi reads can be performed with **GenDx NGSengine software**
SAMPLE SETUP RECOMMENDATIONS FOR SEQUENCING HLA AMPLICON LIBRARIES

SMRT Link Sample Setup provides guidance on how to anneal the sequencing primer, bind the sequencing polymerase and clean up HLA amplicon samples for loading on PacBio sequencing systems.

### SMRT Link Sample Setup Conditions for Primer Annealing and Polymerase Binding

<table>
<thead>
<tr>
<th>SMRT LINK VERSION</th>
<th>SAMPLE SETUP PARAMETER</th>
<th>SEQUEL SYSTEM</th>
<th>SEQUEL II AND IIE SYSTEMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>v10.0</td>
<td>Application</td>
<td>N/A</td>
<td>Select ‘≥3 kb Amplicons’</td>
</tr>
<tr>
<td>v9.0 / v10.0</td>
<td>Sequencing Primer</td>
<td>Sequencing Primer v4</td>
<td>Sequencing Primer v4</td>
</tr>
<tr>
<td></td>
<td>Primer:Template Ratio</td>
<td>20:1</td>
<td>20:1</td>
</tr>
<tr>
<td></td>
<td>Binding Kit</td>
<td>Sequel Binding Kit 3.0</td>
<td>Sequel II Binding Kit 2.0</td>
</tr>
<tr>
<td></td>
<td>Polymerase:Template Ratio</td>
<td>30:1</td>
<td>30:1</td>
</tr>
<tr>
<td></td>
<td>Loading Concentration (pM)</td>
<td>5</td>
<td>50 – 60</td>
</tr>
</tbody>
</table>

### SMRT Link Run Design Settings and Recommended Sequencing Run Parameters

<table>
<thead>
<tr>
<th>SMRT LINK VERSION</th>
<th>RUN DESIGN PARAMETER</th>
<th>SEQUEL SYSTEM</th>
<th>SEQUEL II AND IIE SYSTEMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>v10.0</td>
<td>Application</td>
<td>N/A</td>
<td>Select ‘≥3 kb Amplicons’</td>
</tr>
<tr>
<td></td>
<td>Generate HiFi Reads</td>
<td>N/A</td>
<td>Select ‘On-Instrument’ or ‘In SMRT Link’</td>
</tr>
<tr>
<td>v9.0</td>
<td>Sequencing Mode</td>
<td>Select ‘CCS Reads’</td>
<td>Select ‘CCS Reads’</td>
</tr>
<tr>
<td></td>
<td>Generate CCS Data</td>
<td>Select ‘Yes’ to enable auto-CCS analysis</td>
<td>Select ‘Yes’ to enable auto-CCS analysis</td>
</tr>
<tr>
<td>v9.0 / v10.0</td>
<td>Pre-Extension Time (hours)</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Movie Collection Time (hours)</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
PacBio recommends using GenDx’s NGSengine software for HLA typing.

1. Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel Ile System
   → In SMRT Link, recommend setting Minimum CCS Read Length = 3000 bp

2. Utilize SMRT Link Demultiplex Barcodes analysis application to separate HiFi reads by sample barcode
   → Barcode FASTA files for demultiplexing can be downloaded from PacBio’s Multiplexing website

3. Utilize GenDX NGSengine software to perform HLA Typing analysis of de-multiplexed HiFi data
   → Recommended Input: ≥100 HiFi reads per HLA locus
   → NGSengine accepts as input batches of demultiplexed FASTQ files containing HiFi sequencing data for 1 or more pooled HLA loci.
   → HLA locus assignment and HLA typing for each demultiplexed sample dataset can be performed on a Windows-based laptop computer.

An example demultiplexed HLA dataset analyzed using NGSengine is available for download from PacBio (Link)

https://www.gendx.com/product_line/ngsengine/
EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS

A. HLA Amplicon Generation Using GenDX NGSgo-MX6-1 Kit

PCR PRODUCT YIELDS FOR 8 HUMAN gDNA SAMPLES AMPLIFIED USING THE GenDX NGSgo-MX6-1 KIT.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>NIST ID</th>
<th># of Replicate PCR Reactions</th>
<th>Purified PCR Product Conc. (ng/µl)</th>
<th>Purified PCR Product Volume (µl)</th>
<th>Total Mass of Purified PCR Products (ng)</th>
<th>Mass of Purified PCR Product per Reaction (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA12878</td>
<td>HG001</td>
<td>4</td>
<td>228</td>
<td>16</td>
<td>3,648</td>
<td>912</td>
</tr>
<tr>
<td>NA24385</td>
<td>HG002</td>
<td>4</td>
<td>250</td>
<td>16</td>
<td>4,000</td>
<td>1,000</td>
</tr>
<tr>
<td>NA24149</td>
<td>HG003</td>
<td>4</td>
<td>240</td>
<td>16</td>
<td>3,840</td>
<td>960</td>
</tr>
<tr>
<td>NA24143</td>
<td>HG004</td>
<td>4</td>
<td>221</td>
<td>16</td>
<td>3,392</td>
<td>848</td>
</tr>
<tr>
<td>NA24631</td>
<td>HG005</td>
<td>4</td>
<td>250</td>
<td>16</td>
<td>4,000</td>
<td>1,000</td>
</tr>
<tr>
<td>NA24695</td>
<td>HG007</td>
<td>4</td>
<td>252</td>
<td>16</td>
<td>4,032</td>
<td>1,008</td>
</tr>
<tr>
<td>NA06896</td>
<td>N/A</td>
<td>4</td>
<td>254</td>
<td>16</td>
<td>4,064</td>
<td>1,016</td>
</tr>
<tr>
<td>C1-218</td>
<td>N/A</td>
<td>4</td>
<td>230</td>
<td>16</td>
<td>3,680</td>
<td>912</td>
</tr>
</tbody>
</table>

* For each PCR reaction, 200 ng of input gDNA per sample was used. The fragment size of the input genomic DNA samples ranged from 22 kb to 147 kb (mode).

- Four (4) replicate PCR reactions were performed for each human gDNA sample (input gDNA size mode ~22 kb – 147 kb).
- For each sample, replicate PCR reaction products were pooled and purified with 0.6X AMPure PB beads.
- Bioanalyzer sizing QC results are consistent with expected range of PCR amplicon sizes (~3.1 – 5.7 kb) for these HLA samples.
EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS (CONT.)

B. HLA Amplicon SMRTbell Library Construction Using SMRTbell Express Template Prep Kit 2.0

96-PLEX HLA SMRTBELL LIBRARY CONSTRUCTION YIELD

<table>
<thead>
<tr>
<th>SMRTbell Library</th>
<th>Number of Pooled Samples</th>
<th>Input Mass of PCR DNA per Sample for Library Construction</th>
<th>Total Input Mass</th>
<th>Purified Library Conc. (ng/µl)*</th>
<th>Purified Library Volume (µl)</th>
<th>Purified Library Mass (ng)</th>
<th>Library Construction Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-Plex HLA Library</td>
<td>96</td>
<td>150</td>
<td>14,400</td>
<td>135</td>
<td>96</td>
<td>12,960</td>
<td>90</td>
</tr>
</tbody>
</table>

* The final 96-plex HLA SMRTbell library was purified using two rounds of 0.6X AMPure PB bead purification at the end of the procedure

- Each of eight (8) human gDNA samples that was amplified using the GenDX NGSgo-MX6-1 kit was symmetrically barcoded with 12 unique Barcoded Overhang Adapters to generate a 96-plex pooled HLA SMRTbell library for sequencing on a Sequel II System

- Mean size of the final purified 96-plex HLA SMRTbell library was ~5700 bp
EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS (CONT.)

C. HLA Amplicon SMRTbell Library Primary Sequencing Performance Metrics (Sequal II System)

PRIMARY RUN STATISTICS* FOR A 96-PLEX HLA SMRTBELL LIBRARY

<table>
<thead>
<tr>
<th>SMRTBELL LIBRARY</th>
<th>TOTAL BASES (Gb)</th>
<th>UNIQUE MOLECULAR YIELD (Gb)</th>
<th>MEAN POLYMERASE RL (bp)</th>
<th>POLYMERASE RL N50 (bp)</th>
<th>MEAN LONGEST SUBREAD (bp)</th>
<th>LONGEST SUBREAD N50 (bp)</th>
<th>P0 (%)</th>
<th>P1 (%)</th>
<th>P2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-Plex HLA Library</td>
<td>269.72</td>
<td>45.57</td>
<td>51900</td>
<td>114769</td>
<td>9797</td>
<td>29150</td>
<td>32.3</td>
<td>65</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Sequal II System Chemistry 2.0; 54 pM on-plate loading concentration; 20-hour movie collection time; 1.1-hour pre-extension time

IRLD Plot heat map is consistent with expected range of insert sizes (~3.1 – 5.7 kb) for this pooled HLA amplicon library

* Read lengths, reads/data per SMRT Cell and other sequencing performance results vary based on sample quality/type and insert size.
D. HLA Amplicon SMRTbell Library CCS Analysis and Barcode Demultiplexing Results (Sequel II System)

**96-PLEX HLA SMRTBELL LIBRARY CCS ANALYSIS AND BARCODE DEMULTIPLEXING RESULTS**

<table>
<thead>
<tr>
<th>ANALYSIS</th>
<th>ANALYSIS METRIC</th>
<th>VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCS</td>
<td>HiFi (≥Q20 CCS) Reads</td>
<td>2,229,796</td>
</tr>
<tr>
<td></td>
<td>Unique Barcodes Detected</td>
<td>96</td>
</tr>
<tr>
<td>Demultiplex Barcodes</td>
<td>Total Barcoded HiFi Reads</td>
<td>2,162,071</td>
</tr>
<tr>
<td></td>
<td>Barcode Recovery Rate</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>Mean Barcoded HiFi Reads per Sample</td>
<td>22,521</td>
</tr>
<tr>
<td></td>
<td>Max. Barcoded HiFi Reads per Sample</td>
<td>33,036</td>
</tr>
<tr>
<td></td>
<td>Min. Barcoded HiFi Reads per Sample</td>
<td>12,045</td>
</tr>
</tbody>
</table>

- Mean HiFi Read Length Plot is consistent with expected range of insert sizes (~3.1 – 5.7 kb) for this pooled HLA amplicon library.

*. CCS and barcode demultiplexing analyses were performed with SMRT Link v9.0.

Sequel II System Chemistry 2.0; 54 pM on-plate loading concentration; 20-hour movie collection time; 1.1-hour pre-extension time
EXAMPLE WORKFLOW:  96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS (CONT.)

D. HLA Read Depth Analysis Using GenDX NGSengine (Sequel II System)

- HLA locus DPB1 generated the lowest depth of coverage with sample C1-218
  - Note:  C1-218 gDNA sample QC showed the highest degree of DNA fragmentation with a size distribution mode of 22 kb, whereas other gDNA samples included in this dataset showed a starting modal size range from ~27 – 147 kb.
  - DPB1 is the longest HLA amplicon (5.7 kb) amplified using the GenDX NGSgo-MX1 kit.

To help ensure adequate read coverage, particularly for DPB1 alleles, we recommend pooling a maximum of 96 samples per Sequel II System SMRT Cell 8M (and a maximum of 46 samples per Sequel System SMRT Cell 1M) for HLA sequencing.
D. HLA Typing Results Using GenDx NGSengine Software

Sample: demultiplex.24143-HG004

Full typing result

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>CWD 1</th>
<th>CWD 2</th>
<th>Review status</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>01:01:01:01</td>
<td>33:01:01:01</td>
<td>C</td>
<td>C</td>
<td>Not reviewed</td>
</tr>
<tr>
<td>HLA-B</td>
<td>14:02:01:01</td>
<td>35:08:01:01</td>
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Continuous red line marking indicates the HLA data are **fully phased**

An example demultiplexed HLA dataset analyzed using NGSengine is available for download from PacBio (Link)